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Osteoprotegerin Ligand Modulates Murine Osteoclast Survival *in Vitro* and *in Vivo*

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Osteoprotegerin ligand (OPGL) targets osteoclast precursors and osteoclasts to enhance differentiation and activation, however, little is known about OPGL effects on osteoclast survival. *In vitro*, the combination of OPGL + colony-stimulating factor-1 (CSF-1) is required for optimal osteoclast survival. Ultrastructurally, apoptotic changes were observed in detached cells and culture lysates exhibited elevated caspase 3 activity, particularly in cultures lacking CSF-1. DEVD-FMK (caspase 3 inhibitor) partially protected cells when combined with OPGL, but not when used alone or in combination with CSF-1. CSF-1 maintained NF-κB activation and increased the expression of bcl-2 and bcl-X_L mRNA, but had no effect on JNK activation. In contrast, OPGL enhanced both NF-κB and JNK kinase activation and increased the expression of c-src, but not bcl-2 and bcl-X_L mRNA. These data suggest that aspects of both OPGL's and CSF-1's signaling/survival pathways are required for optimal osteoclast survival. In mice, a single dose of OPG, the OPGL decoy receptor, led to a >90% loss of osteoclasts because of apoptosis within 48 hours of exposure without impacting osteoclast precursor cells. Therefore, OPGL is essential, but not sufficient, for osteoclast survival and endogenous CSF-1 levels are insufficient to maintain osteoclast viability in the absence of OPGL. ***(Am J Pathol 2000, 157:435–448)***

Osteoclasts mediate the resorption component of bone modeling and remodeling, which together are pivotal to the formation and maintenance of the mammalian skeleton. These specialized members of the monocyte-macrophage family arise from hematopoietic precursors with the location and magnitude of their activity guided by cells that surface the bone matrix. The bone lining cells, which include osteoblasts, endosteal, and periosteal lining cells, seem to mediate the local, systemic, physiological, and/or pathological stimuli impinging on them and consequently provide molecular signals that eventuate in osteoclast-mediated bone resorption.[1,2]

In the mouse, and likely in humans as well, two factors seem to be critical for osteoclast differentiation and activation.[3,4] The monocyte lineage colony stimulating factor, CSF-1, was discovered to be one of these factors based on molecular analysis of the op/op osteopetrotic mouse. These mice, which are severely osteoclast-deficient, have a stop codon mutation in the CSF-1 coding region that abrogates the production of active protein.[5] The second factor is osteoprotegerin ligand (OPGL),[3,6] a novel tumor necrosis factor (TNF) family member that has also been described as TRANCE, RANKL, and ODF.[6–8] This long sought after factor complements CSF-1 to stimulate the formation of osteoclasts from hematopoietic precursors in the absence of other cytokines or colony factors.[3,6] Both OPGL and CSF-1 are made by stromal cells and osteoblasts and their production is regulated by factors known to modulate bone resorption activity.[6] Osteoblasts also secrete a soluble decoy receptor for OPGL, OPG,[9,10] which can down-regulate the effects of OPGL by sequestering it from the OPGL cell-surface receptor.[11,12]

OPGL can also stimulate the activities of mature osteoclasts.[13,14] Using preformed osteoclasts from neonatal rat long bones, we have shown that OPGL stimulates bone resorption in a pit-forming assay and rapidly induces the formation of actin ring structures, which are required for bone resorption to occur. Finally, injections of OPGL in normal mice lead to hypercalcemia too rapidly for osteoclast formation to occur indicating that OPGL targets pre-existing osteoclasts in bone.

The above studies have clearly shown that OPGL plays a key role in the differentiation of osteoclasts from hematopoietic precursors as well as the activation of mature osteoclasts. What is not clear is the importance and potential mechanisms of OPGL in the determination of osteoclast survival once the mature cell has formed. Recent reports using neonatal rat osteoclasts[14] or purified cultured-murine osteoclasts[15] suggest that OPGL/ODF/TRANCE alone is sufficient to maintain osteoclast viability *in vitro* with NF-κB activation playing some role in this effect. In this study, we have examined the effects of

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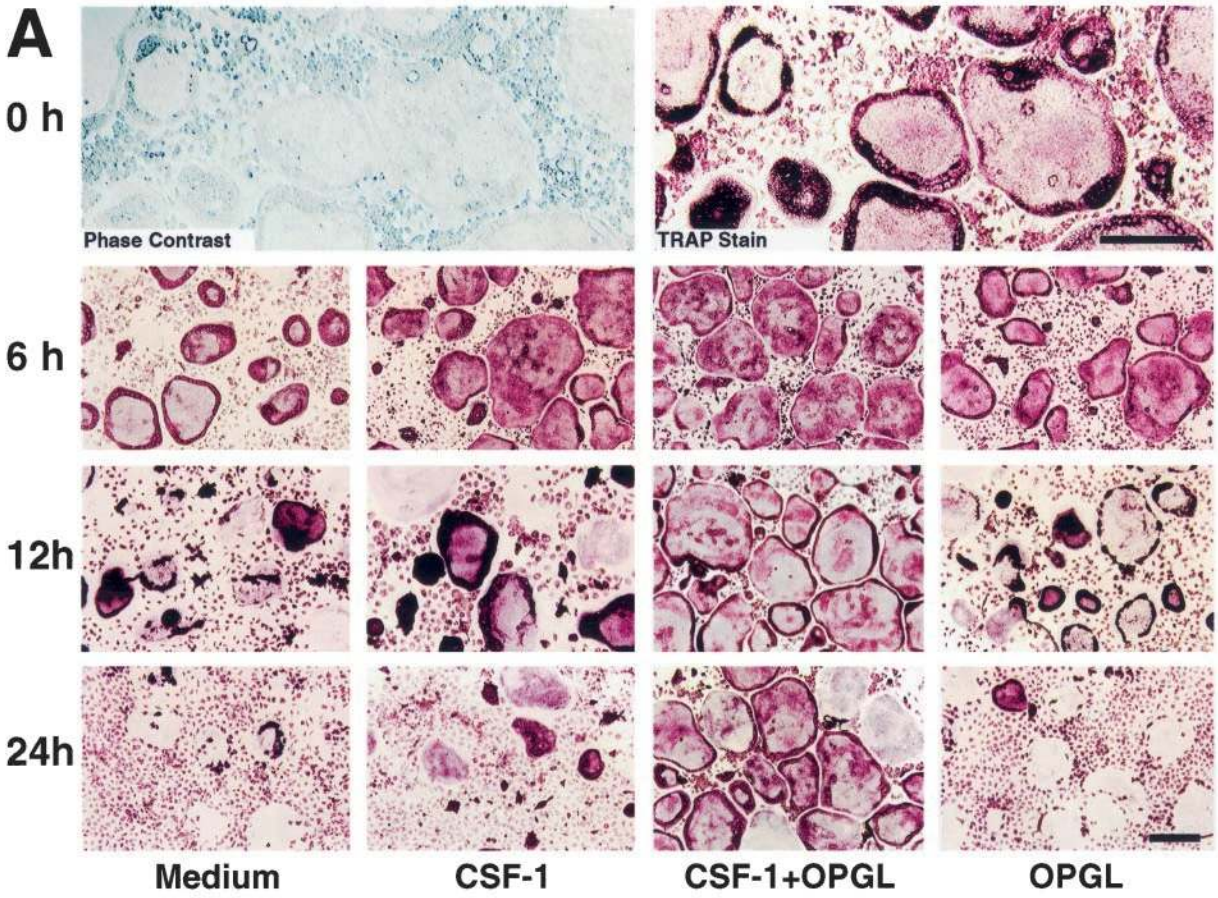


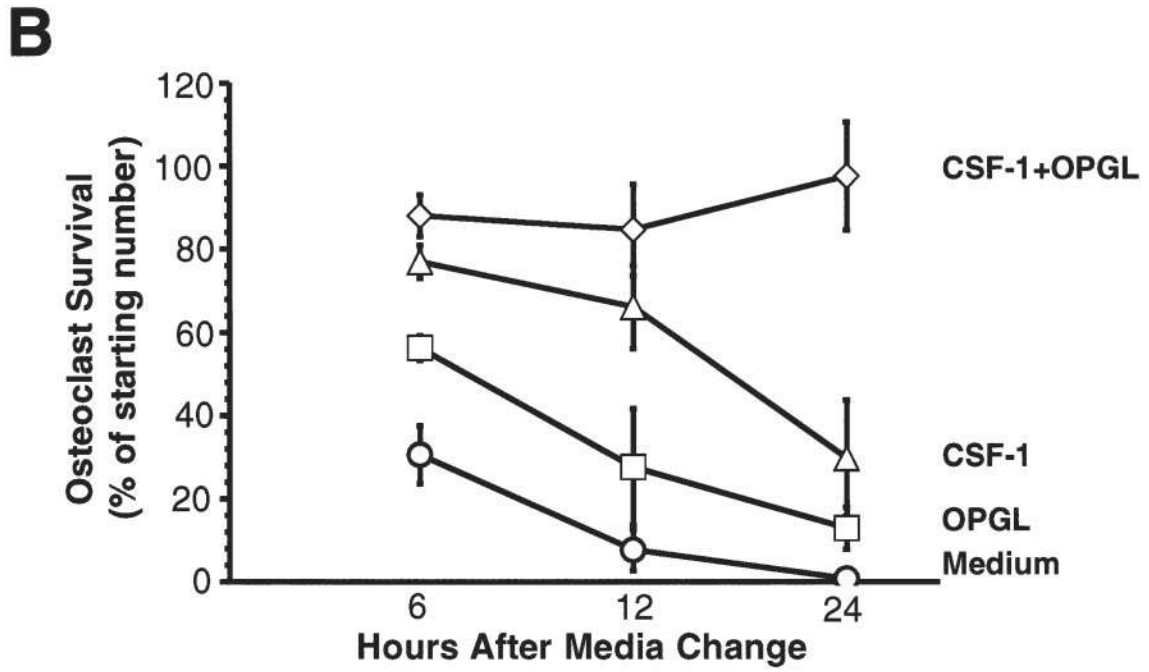


Figure 1. OPGL and CSF-1 effects on *in vitro* osteoclast survival. Osteoclasts were developed *in vitro* from bone marrow cells using media supplemented with CSF-1 and OPGL as described. After 5 to 6 days, large osteoclast-like cells were obvious by phase contrast microscopy and the wells were rinsed and the media replaced with medium alone, CSF-1 (30 ng/ml), OPGL alone (100 ng/ml), or CSF-1 + OPGL (100 ng/ml). Wells were stained for TRAP activity at T = 0 and at 6, 12, and 24 hours (h) after the media was changed. **A:** Representative photomicrographs of the various conditions taken at the indicated times are shown. Scale bars, 200 μm. **B:** Osteoclasts were counted in triplicate wells in the various conditions and the results normalized to the number present at time = 0, which was 253 ± 19. The results from three separate experiments were pooled and the data represent the mean ± SD, n = 9. Note that the combination of CSF-1 + OPGL maintains osteoclast numbers at starting levels throughout the 24-hour observation period.

OPGL and its withdrawal on the survival of osteoclasts both *in vitro* and *in vivo*. *In vitro*, removal of both OPGL and CSF-1 from bone marrow cultures where CSF-1 + OPGL had driven osteoclast differentiation quickly leads to a significant reduction in osteoclast numbers because of apoptosis. Withdrawal of either CSF-1 or OPGL alone also leads to osteoclast apoptosis with CSF-1 removal leading to a more rapid disappearance of osteoclasts indicating that neither CSF-1 nor OPGL alone are sufficient for optimal osteoclast survival. The apoptotic mechanism seems to involve at least two distinct pathways, one of which is caspase 3-mediated. *In vivo*, blockade of either administered or endogenous OPGL using a single, high dose of recombinant OPG delivered intravenously leads to the disappearance of virtually all of the osteoclasts along both the endosteal and periosteal surfaces throughout a 48-hour period. These findings indicate that OPGL is critical for osteoclast survival *in vivo* in normal animals and that its depletion leads to programmed cell death.

Materials and Methods

In Vitro *Osteoclast Cultures*

Osteoclast forming cultures were established as described[3] using bone marrow from C3H/HeN mice (Charles River Laboratories, Wilmington, MA) as a source of osteoclast progenitors. The media used to drive osteoclast differentiation was α-minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (termed basal media) that was supplemented with murine recombinant CSF-1 (30 ng/ml, R&D Systems, Minneapolis, MN) and murine recombinant OPGL (158 to 316, 100 ng/ml).[3] When osteoclasts appeared, the media was removed and the plates gently rinsed. The media was then replaced with basal media, or basal media supplemented with CSF-1, OPGL, or CSF-1/OPGL. In some experiments, the caspase 3 or nonspecific caspase inhibitors, DEVD-FMK or zVAD-FMK (100 μmol/L; Enzyme Systems Products, Dublin, CA), were also added as indicated. At various times, the supernatants were collected and the adherent layer stained cytochemically for tartrate resistant acid phosphatase (TRAP) activity as described.[11] Supernatant cytospins were prepared using a Shandon Cytospin 3 cytocentrifuge (Shandon Scientific, Ltd., Runcorn, UK) set at 200 rpm for 3 minutes and then stained with Wright's Giemsa or TRAP stains. In the osteoclast-forming assay using osteoclast progenitors from OPG or saline-treated mice, various concentrations of OPGL were used together with CSF-1 (30 ng/ml) and a solution assay that quantitates TRAP culture activity was used to assess osteoclast differentiation.[11]

Ultrastructural Analysis

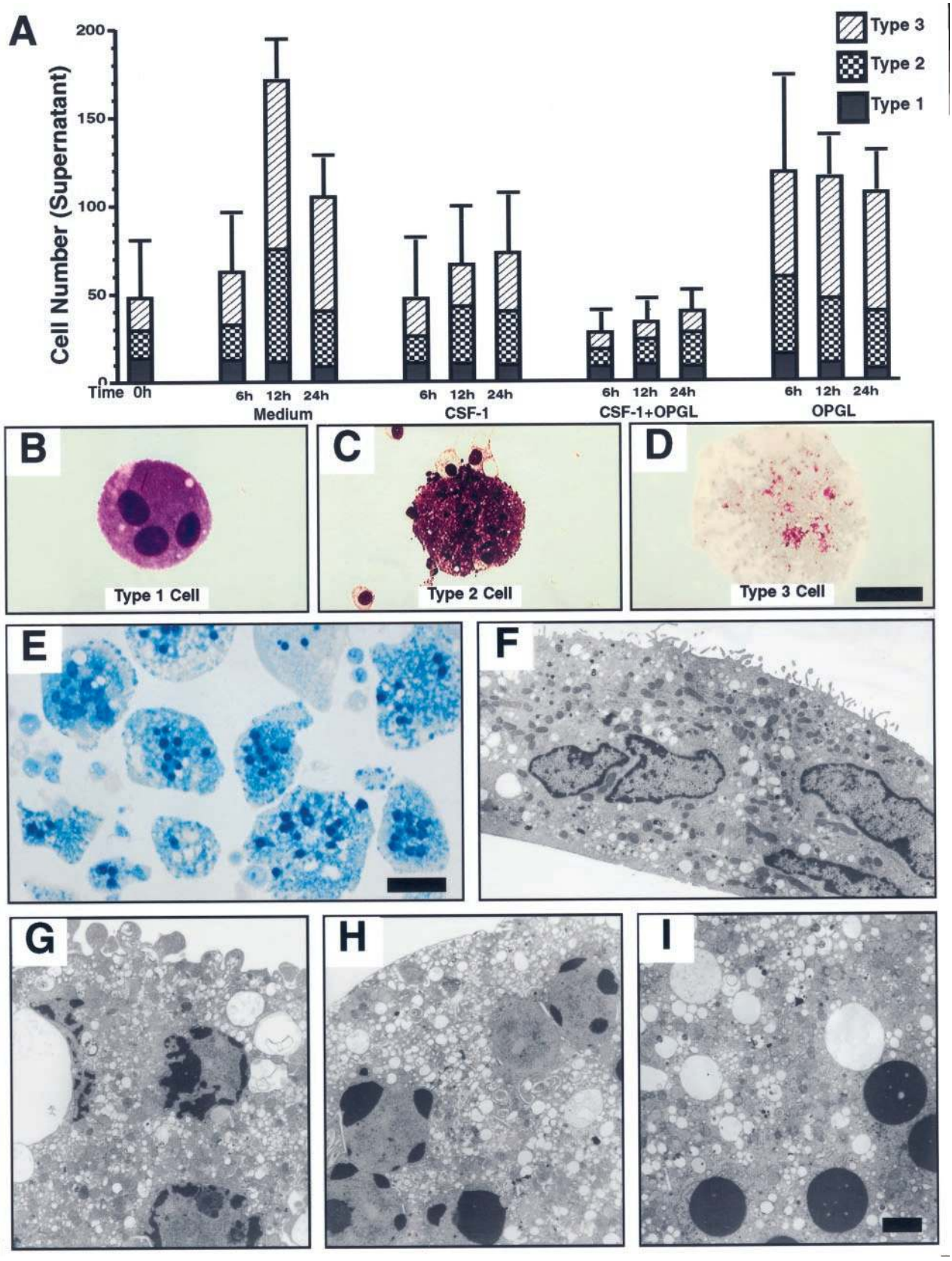
Osteoclast cultures were established on polyethylene terephthalate track-etched membranes (0.4 μm; Becton Dickinson, Franklin Lakes, NJ) and manipulated as above. At various times, the supernatants were collected and the detached cells gently pelleted (5 minutes at 500 relative centrifugal force) and resuspended in 3% glutaraldehyde in cold 0.1 mol/L sodium cacodylate buffer at pH 7.4. After 24 hours fixation at 4°C, the cells were rinsed in buffer, postfixed for 1 hour with 1% aqueous osmium tetroxide, rinsed in water, and transferred to 2-ml microcentrifuge tubes. The adherent cells were fixed *in situ* for 24 hours, rinsed, then postfixed with 1% aqueous osmium tetroxide. The cells were gently dislodged using a rubber policeman and then pelleted in 2-ml microcentrifuge tubes. The pellets were dehydrated in ethanol and embedded in an epoxy resin. Light microscopic examination of all embedded materials was conducted on toluidine blue-stained, 1-μm sections. Ultra-thin sections were collected on 200-mesh copper grids and contrast-enhanced with uranyl acetate and lead citrate before examination on a Philips CM120 transmission electron microscope.

Molecular Analyses

The following sequences were generated by reverse transcription-polymerase chain reaction from mouse thymus (bcl-2 and bcl-X_L) or mouse osteoclasts (c-src)[3] and cloned into the transcription vector pGEM-T (Promega, Madison, WI): bcl-2 (GenBank: M16506, bases 1846 to 2264), bcl-X_L (GenBank: L35049, bases 527 to 735), and c-src (GenBank: M17031, bases 259 to 431). A 105-bp murine cyclophilin probe (Ambion, Austin, TX) was used as an internal control. After cloning, the vectors were linearized, and radiolabeled-antisense transcript was synthesized using SP6 or T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) and [^{32}P]rUTP (800 Ci/mol; Amersham, Arlington Heights, IL). The probes were purified on a 6% polyacrylamide/7 mol/L urea gel. The caspase probes were synthesized from the mAPO-1 template (Pharmingen, San Diego, CA). The RNase protection assay was performed using the RPA II kit (Ambion, Inc., Austin, TX) and 15 μg (for bcl-2 and bcl-X_L), 10 μg (for caspases), or 5 μg (for c-src) of total RNA from each sample. Quantitation was performed with a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The integrated volume of the probe band and internal control band was calculated and the ratio of the two was averaged.

Caspase Assays

Cytosolic extracts of cells were prepared from osteoclast cultures as described.[16] For caspase activity assays, 20 to 40 μg of cell lysates were diluted 10-fold in assay buffer (50 mmol/L HEPES, pH 7.5, 10% sucrose, 100 mmol/L NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) containing 50 μmol/L substrate and incubated at room temperature for up to 1 hour. The substrates tested included, YVAD-AFC (caspase 1), DEVD-AFC (caspase 3), LETD-AFC (caspase 8), and IETD-AFC (caspase 9) and were from Enzyme Systems Products (Dublin, CA). The release of AFC was measured in


A
Cell Number (Supernatant)
200
150
100
50
0
Type 3
Type 2
Type 1
Time 0h
6h 12h 24h
Medium
6h 12h 24h
CSF-1
6h 12h 24h
CSF-1+OPGL
6h 12h 24h
OPGL
B
Type 1 Cell
C
Type 2 Cell
D
Type 3 Cell
E
F
G
H
I

a Cytofluor II, fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, MA) using an excitation filter of 400/30 nm and an emission filter of 508/20 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as described previously.[16] The mouse anti-caspase 3 antibody was obtained from Transduction Laboratories (Catalog no. C76920, Lexington, KY). Densitometry on autoradiograms was performed using a Fluorchem 8000 and AlphaEase Software (AlphaInnotech Corp., San Leandro, CA).

NF-κB and JNK Kinase Assays

Osteoclasts cultures were rinsed and then treated for 30 minutes with temperature (37°C) and CO_2-equilibrated basal media alone, or basal media supplemented with CSF-1 (30 ng/ml), OPGL (100 ng/ml), or CSF-1 + OPGL. For NF-κB activation, gel retardation assays were performed using the Gel Shift Assay Kit (Stratagene, La Jolla, CA) using the manufacturer's recommended conditions to detect NF-κB activity. For endogenous JNK kinase activity, culture lysates were prepared and assayed as previously described.[17,18] The GST-cjun (1 to 143) substrate was purified with a commercially available purification module (Pharmacia Biotech, Piscataway, NJ) using the manufacturer's recommended conditions.

In Vivo *Studies*

C3H/HeN male mice, 6 to 7 weeks old, were injected subcutaneously with either saline or OPGL (1 mg/kg/day) for 7 days. Three hours after the last treatment, each group was further subdivided into two groups. These subgroups were administered a single dose of either saline or OPG (10 mg/kg) intravenously. The recombinant OPG used was a truncated human molecule lacking the c-terminal heparin binding domain that was fused at the amino terminus to a human Fc sequence.[11] At time = 0 (time of OPG treatment) and at various times up to 192 hours, groups of animals were sacrificed and the femurs and tibiae were collected.

Histology/Immunohistochemistry

The femurs and tibiae were processed into paraffin as described.[11] Bone sections were stained with hematoxylin and eosin and were also subjected to immunohistochemistry using cathepsin K antibodies.[19] For immunohistochemistry, sections (4 μm) were blocked with CAS Block (Zymed Laboratories, San Francisco, CA) and incubated with affinity-purified anti-cathepsin K antibodies (10 μg/ml). A biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame CA) was used as the secondary antibody. The tertiary reagent was an alkaline phosphatase conjugated avidin-biotin complex (Vector Laboratories) and Vector Red (Vector Laboratories) was the substrate used to develop the staining reactions. All sections were lightly counterstained with hematoxylin.

Histomorphometry

Osteoclast numbers were assessed in the distal 5 mm of the femur from the midshaft to the metaphyseal side of the growth plate on cathepsin K-immunostained sections using image analysis (MetaMorph; Universal Imaging Co., West Chester, PA) and were expressed in terms of osteoclasts/total tissue area. Apoptotic osteoclasts were identified in cathepsin K-stained sections and were expressed as apoptotic osteoclasts/total tissue area. The total tissue area examined in the femur sections averaged 4.64 ± 0.504 mm^2 (X ± SD, $n = 200$).

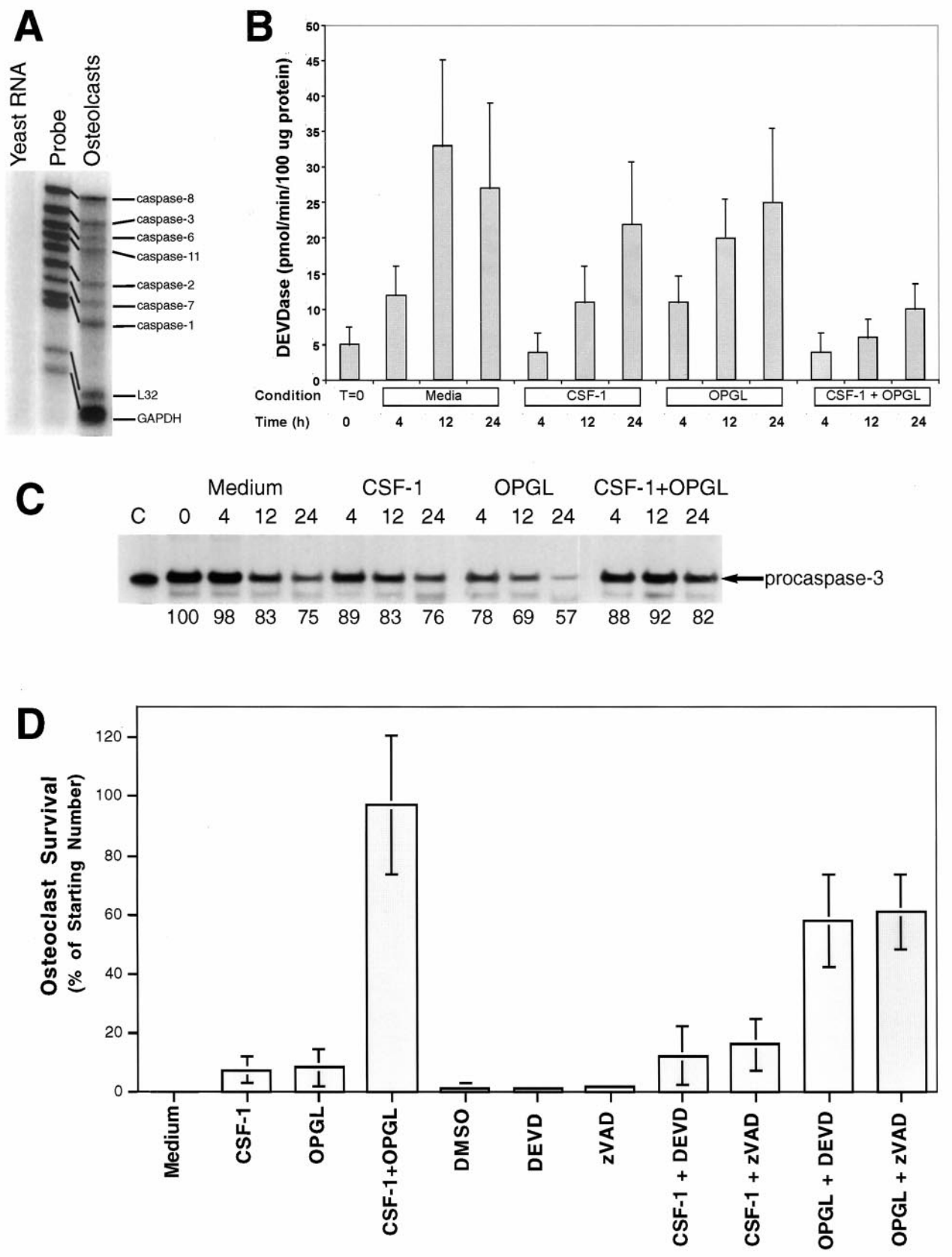
Statistical Analysis

The *in vivo* data were analyzed by analysis of variance using a computer-based statistical program (Statview; Abacus Concepts Inc., Berkeley, CA).

Results

To determine the role of OPGL in osteoclast survival, bone marrow cultures treated with OPGL/CSF-1 were followed until osteoclast-like cells became apparent (see Figure 1 for example) after which the media was replaced as indicated. In the presence of media alone, osteoclasts rapidly disappeared from the cultures with observable declines seen by 6 hours (Figure 1) with <5% remaining at 24 hours. When CSF-1 alone was re-added, osteoclasts still disappeared from the cultures, but the rate was slower than that observed in the media-alone group with significant differences present only by the 24-hour time point where the osteoclast number was ~30% of the starting number. Surprisingly, re-addition of OPGL did not prevent the disappearance of osteoclasts, because their rate of disappearance was intermediate between the CSF-1-alone and medium-alone conditions. However,

Figure 2. Cyto- and ultrastructural morphology of degenerating cells in culture supernatants from osteoclast cultures. Osteoclast cultures were established and manipulated as described in Figure 1. Supernatants were collected and cytospins were prepared. Large multinucleated cells with intact nuclei (by light microscopy), large cells with fragmented nuclear debris, and large spherical cell fragments with no apparent nuclei were observed in culture supernatants and are indicated as type 1 (**B**), type 2 (**C**), and type 3 cells (**D**), respectively. The scale bar in **D** represents 50 μm and applies to **B–D**. These three types were quantitated across three experiments, combined and are presented in **A** as the mean ± SD ($n = 9$). The error bar represents the SD of the summed events. Note that the media alone and OPGL alone groups had the highest level of detached cells with degenerative changes and that these debris were increased as early as 6 hours when compared to CSF-1 + OPGL. **E:** A toluidine blue, 1-μm plastic section of supernatant osteoclasts with apoptotic features are shown. The supernatant came from an osteoclast culture 6 hours after being placed in basal media. **F:** An electron micrograph of a viable osteoclast harvested from the culture membrane surface at the time of media changing is shown. **G–I:** Osteoclasts with a progression of apoptotic features are shown. **G:** A cell with nuclear chromatin condensation and margination together with cell membrane blebbing is shown. **H:** A cell with more extensive chromatin condensation and margination, loss of membrane blebs, and more extensive cytoplasmic vacuolization is shown. **I:** A cell with extensive nuclear fragmentation is shown. The features shown in **G–I** were from cells in **E**. The scale bars in **E** and **I** represent 25 and 2 μm, respectively, and the scale bar in **I** applies to **F–I**.


A
Yeast RNA
Probe
Osteolcasts
caspase-8
caspase-3
caspase-6
caspase-11
caspase-2
caspase-7
caspase-1
L32
GAPDH
B
DEVDase (pmol/min/100 ug protein)
Condition
T=0
Media
CSF-1
OPGL
CSF-1 + OPGL
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C
Medium
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CSF-1+OPGL
procaspase-3
100 98 83 75 89 83 76 78 69 57 88 92 82
D
Osteoclast Survival
(% of Starting Number)
Medium
CSF-1
OPGL
CSF-1+OPGL
DMSO
DEVD
zVAD
CSF-1 + DEVD
CSF-1 + zVAD
OPGL + DEVD
OPGL + zVAD

osteoclast numbers were maintained when both OPGL and CSF-1 were re-added.

Three different types of cells or debris were found and quantified in cytospins of culture supernatants (Figure 2, B–D) and all of the debris and cells found were TRAP-positive. The magnitude of detached cells and cell fragments seen in the various groups (Figure 2A) were generally inversely related to the numbers of surviving cells that remained adhered to the culture dishes (Figure 1B). This suggests that the osteoclasts seen after 24 hours in the CSF-1 + OPGL condition were there primarily as a result of improved survival. Electron micrographs (Figure 2) provide ultrastructural evidence that osteoclast numbers decline because of apoptosis. Apoptotic osteoclasts were present in all conditions suggesting that once the cell death program had been initiated, the path(s) of self-destruction could not be altered.

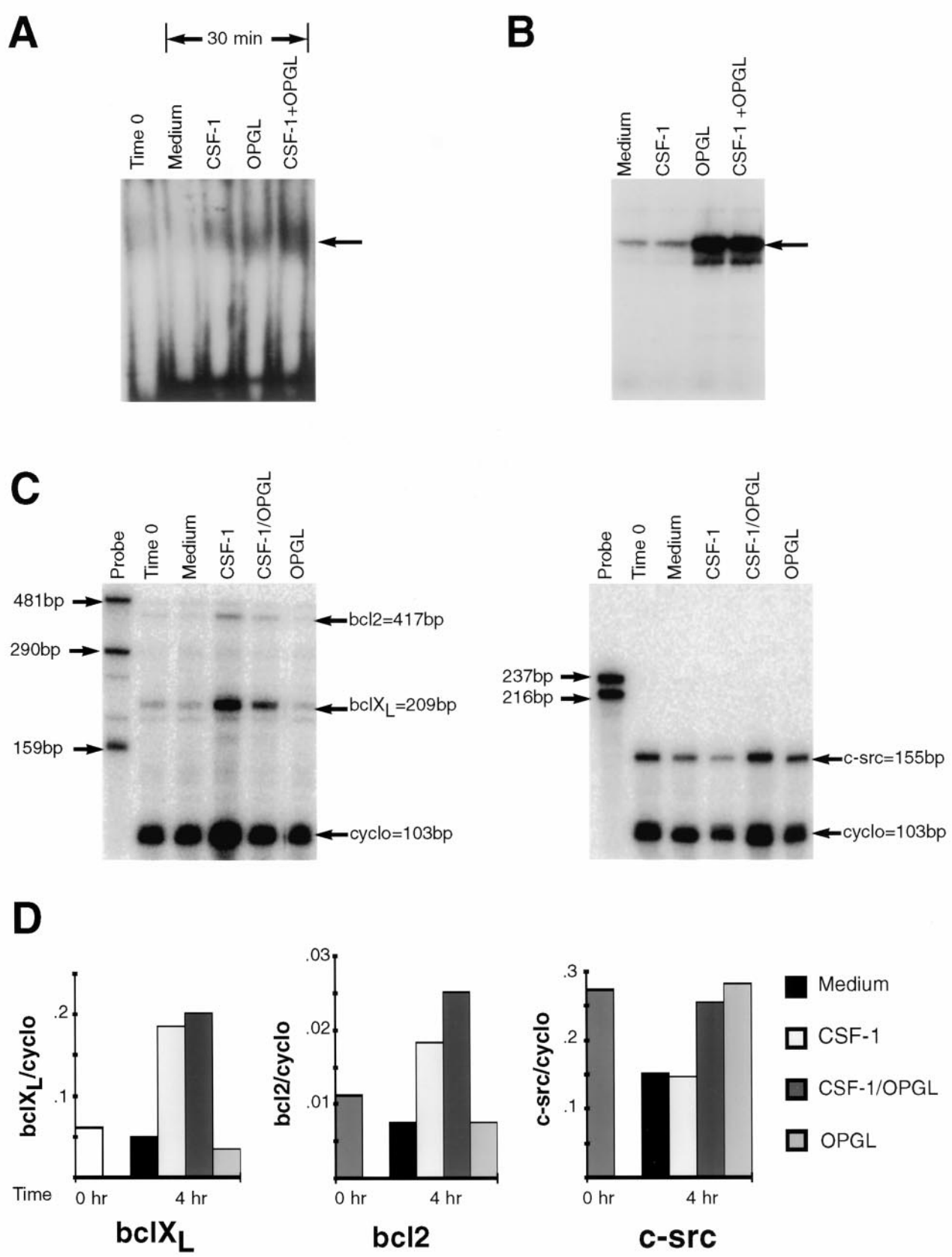
The process of apoptosis frequently involves activation of a cascade of events that are initiated and perpetuated by members of the caspase family.[20] We detected mRNA for caspases 1, 2, 3, 6, 7, 8, and 11 by RNase protection assay (Figure 3A) in total RNA from *in vitro*-derived osteoclasts. Using culture lysates and various caspase substrates including YVAD-AFC, DEVD-AFC, LETD-AFC, or IETD-AFC, which are somewhat selective for caspases 1, 3, 8, or 9, respectively, we find that culture lysates contain enzymatic activity selective for DEVD-AFC (Figure 3B). In all circumstances except for the combination of CSF-1 + OPGL, elevations of caspase 3-like activity were seen by 24 hours with a modest delay for caspase 3-like activity elevations in the CSF-1 alone condition compared to media-alone and OPGL-alone conditions. Western blots of the osteoclast lysates (Figure 3C) demonstrate the presence of procaspase 3 in culture lysates that disappears over time, particularly in the OPGL- and medium-alone conditions. This observation is consistent with activation of procaspase 3 leading to the generation of active caspase 3, which can be difficult to detect.[21] DEVD-FMK and zVAD-FMK were used to block osteoclast apoptosis *in vitro* and neither of these inhibitors blocked apoptosis when used alone (Figure 3D). However, both inhibitors complemented OPGL, but not CSF-1, in improving osteoclast survival. These data suggest that lack of CSF-1 causes caspase 3 activation leading to apoptosis. On the other hand, the loss of OPGL activates a pathway involving a novel caspase or non-caspase enzyme which concomitantly activates caspase 3 either directly or indirectly (Figure 3B) and is insensitive to specific (DEVD-FMK) and nonspecific (zVAD-FMK) caspase inhibitors (Figure 3D).

OPGL has been shown activate both NF-κB and/or JNK kinase in other cell types raising the possibility that these pathways may contribute to osteoclast survival. As shown in Figure 4A, CSF-1 or OPGL produce a modest increase in NF-κB activity in mature osteoclasts after a 30-minute exposure. The combination of OPGL and CSF-1 synergized to produce higher levels of NF-κB binding activity. OPGL, but not CSF-1, also stimulates JNK kinase activity in these cultures as shown in Figure 4B. We have not seen any evidence for an interaction between OPGL and CSF-1 on JNK kinase activation. Hence, in cultures enriched for mature osteoclasts, OPGL alone stimulates both NF-κB and JNK kinase whereas CSF-1 seems to impact only NF-κB activity.

As shown in Figure 4, C and D, osteoclast cultures exposed to CSF-1, whether alone or combined with OPGL, express increased amounts of bcl-X_L mRNA. While bcl-2 mRNA levels were only ~10% of bcl-X_L, CSF-1 treatment again led to increases in bcl-2 mRNA expression similar in magnitude to bcl-X_L mRNA expression. OPGL did not alter the expression of either bcl-2 or bcl-X_L mRNA, nor did it seem to enhance the effect of CSF-1 on the expression of these anti-apoptotic proteins. However, OPGL, but not CSF-1, re-addition did serve to maintain c-src mRNA expression at elevated levels in the presence or absence of CSF-1.

To study the role of OPGL in osteoclast survival *in vivo*, a recombinant form of OPG, the secreted high-affinity OPGL receptor, was used to sequester OPGL, either endogenously expressed or administered, in mice. Groups of mice were treated for 7 days with daily injections of either saline or OPGL (1 mg/kg) subcutaneously.[3] Three hours after the last injection of OPGL or saline, a single large dose of recombinant OPG (10 mg/kg) or saline was administered. After a week of OPGL injections, just before OPG injection, osteoclast numbers were increased approximately twofold compared to control (Figure 5A; Figure 6, A and B). After cessation of OPGL treatments, osteoclast numbers declined slightly throughout the next 48 hours, but not to control levels (Figure 5A; Figure 6D). In contrast, OPGL-treated animals given OPG exhibited a marked, rapid decline in osteoclasts such that by 48 hours essentially none remained (Figure 5A; Figure 6F). There was some lag between OPG administration and osteoclast disappearance as the greatest osteoclast decline was seen between 12 and 24 hours after OPG treatment (Figure 5A). Similar changes were seen in saline-treated animals given OPG (Figure 5A; Figure 6, A and E), but the osteoclast starting number was obviously less. Saline-treated animals challenged

Figure 3. Caspase involvement in osteoclast apoptosis. **A:** Osteoclast culture RNA was screened for caspase mRNA by RNase protection assay as described in the Methods. The bands representing specifically expressed caspase mRNAs are indicated. **B:** Osteoclast lysates were obtained at time = 0 and at the indicated times after media exchange and assayed for caspase 3-like activity using DEVD-AFC. Caspase activity is expressed as the rate of substrate cleavage normalized to protein amount. The values were pooled from two experiments ($n = 6$) and represent the mean ± SD. **C:** Osteoclast lysates from the various conditions taken at the indicated times were assessed for procaspase 3 protein by Western blot analysis. The lane labeled "C" is the RSV-3T3 lysate provided by the manufacturer as a control. Densitometry values are below each lane and are expressed as a percent of the T = 0 value. **D:** Osteoclast cultures were developed as described and the media were exchanged and replaced with CSF-1 (30 ng/ml), OPGL (100 ng/ml), DEVD-FMK (100 μM), zVAD-FMK (100 μM), or the indicated combinations. Twenty-four hours later osteoclast numbers were obtained and then normalized to the starting number and expressed as the percentage of the starting osteoclast number which was 200 ± 54. The values were pooled from two experiments ($n = 6$) and represent the mean ± SD. Osteoclasts express a range of different caspases and lysate analysis show that caspase 3 activation occurs in osteoclasts not containing both CSF-1 and OPGL. Western blot analysis shows disappearance of procaspase 3 consistent with activation, particularly in the media- and OPGL-alone conditions. Caspase inhibition preserves osteoclast viability when combined with OPGL.


A
30 min
Time 0
Medium
CSF-1
OPGL
CSF-1+OPGL
B
Medium
CSF-1
OPGL
CSF-1 +OPGL
C
Probe
Time 0
Medium
CSF-1
CSF-1/OPGL
OPGL
481bp
290bp
159bp
bcl2=417bp
bclX$_L$=209bp
cyclo=103bp
237bp
216bp
c-src=155bp
cyclo=103bp
D
bclX$_L$/cyclo
.2
.1
Time
0 hr
4 hr
bclX$_L$
bcl2/cyclo
.03
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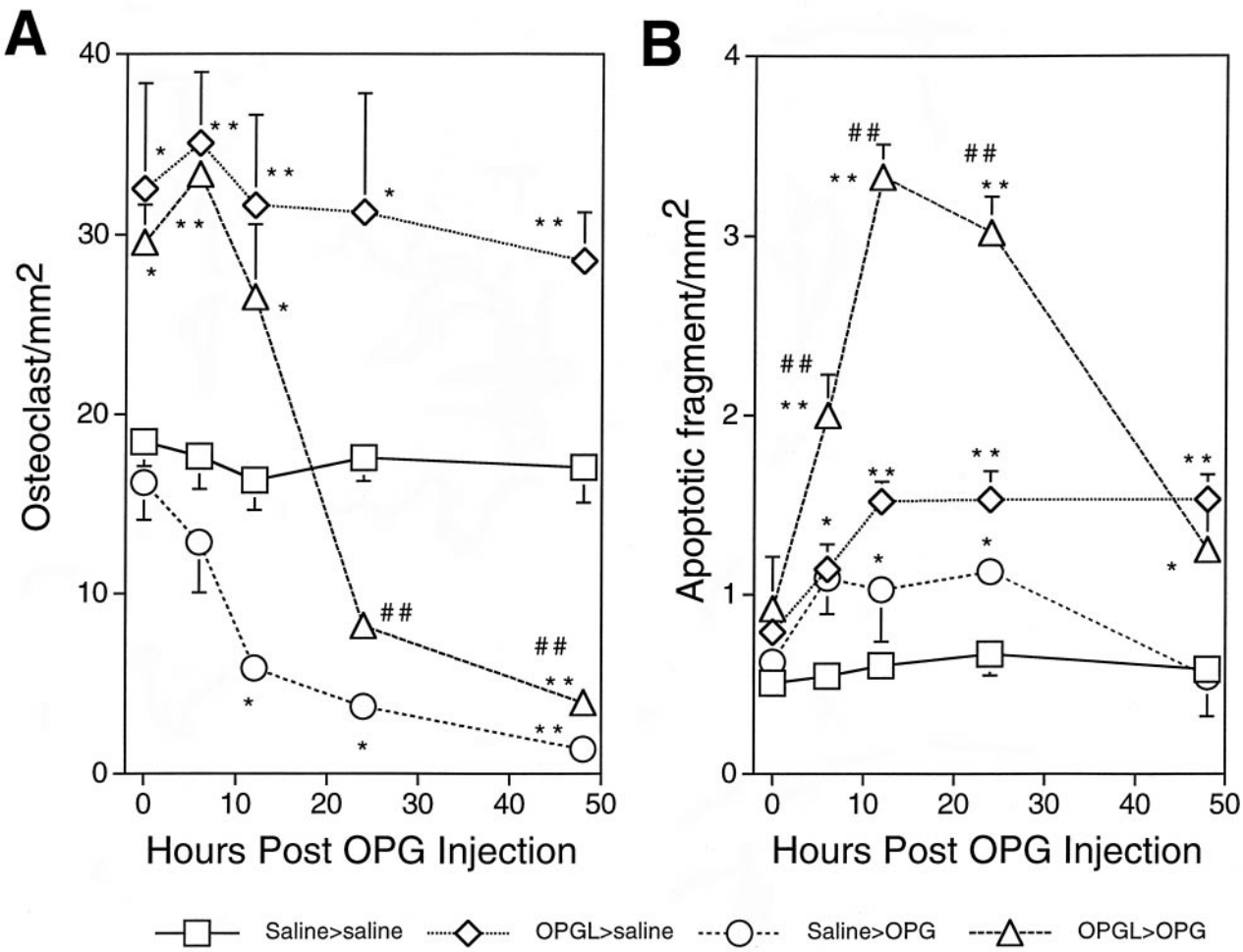


Figure 5. Quantitative histomorphometry of viable and apoptotic osteoclasts in mice treated with OPGL and OPG. Mice were treated with either saline or OPGL (1 mg/kg, SC) for 1 week then, 3 hours after the last injection, a single dose of OPG (10 mg/kg, i.v.) or saline was administered. Histological sections of the distal femur stained by cathepsin K immunohistochemistry from mice sacrificed at 0, 6, 12, 24, and 48 hours after the OPG injection were assessed for viable (**A**) and apoptotic (**B**) osteoclasts. Both measures are expressed as events/tissue area (mm^2) and the data shown are the mean ± SD (n = 5 animals/group). Different from saline → saline group: ***,** $P < 0.05$, ****,** $P < 0.01$; different from OPGL → saline group: **##**, $P < 0.01$.

with saline had stable osteoclast numbers throughout the 48-hour period (Figure 5A; Figure 6C).

Concurrent with the disappearance of osteoclasts was the appearance of apoptotic osteoclasts, which were observed adjacent to bone along both the endosteal and periosteal surfaces (for examples see Figure 7B). In the OPG-treated, OPGL group apoptotic osteoclasts were evident at 6 hours, maximizing at hour 24, and declining thereafter (Figure 5B). In the OPG-treated, saline group maximal osteoclast apoptosis was also seen at 24 hours. Apoptotic osteoclasts were also seen in the OPGL-treated group given saline. In this group, apoptotic osteoclasts become detectable at hour 24 and remain elevated at hour 48. These observations strongly indicate that the removal of OPGL via OPG led to osteoclast apoptosis *in vivo*.

To exclude the possibility that osteoclastogenesis had been permanently disrupted, osteoclast recovery was followed out to 8 days after OPG exposure (10 mg/kg, i.v., single dose) in mice pretreated with either saline or OPGL (1 mg/kg) for 7 days. As shown in Figure 8A, osteoclast counts in OPG-treated animals nadir to nearly 0 at 48 hours and remain very low up to 96 hours after OPG treatment. After 8 days, the OPG-treated groups have slightly increased numbers of osteoclasts relative to controls suggesting that a modest rebound has occurred. In

Figure 4. CSF-1 and OPGL effects on NF-κB and JNK kinase activation and bcl-2, bcl-X_L, and c-src mRNA expression in osteoclast cultures. *In vitro* osteoclast cultures were established as described in Figure 1. For NF-κB (**A**) and JNK kinase (**B**) activation studies, osteoclast cultures were rinsed and then exposed to basal media, OPGL (100 ng/ml), CSF-1 (30 ng/ml), or their combination for 30 minutes. **A:** An electrophoretic mobility shift assay using ^{32}P-labeled double-stranded oligonucleotides containing NF-κB consensus sequences is shown. **B:** JNK immunoprecipitates from the osteoclast cultures were assayed for kinase activity using GST-JUN as a substrate and they were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For bcl-2, bcl-X_L, and c-src mRNA expression total cell RNA was isolated at time = 0 and 4 hours after the media were changed. RNase protection assays were performed. **C:** Phosphorimages show the effect of the various treatments on bcl-2, bcl-X_L (**left**) and c-src mRNA expression (**right**). Quantitative analysis of relative expression levels (normalized to cyclophilin) is shown in **D**. Note that CSF-1 increases NF-κB activation and bcl-X_L and bcl-2 mRNA expression and that OPGL increases both NF-κB and JNK kinase activation and c-src mRNA expression in these cultures.

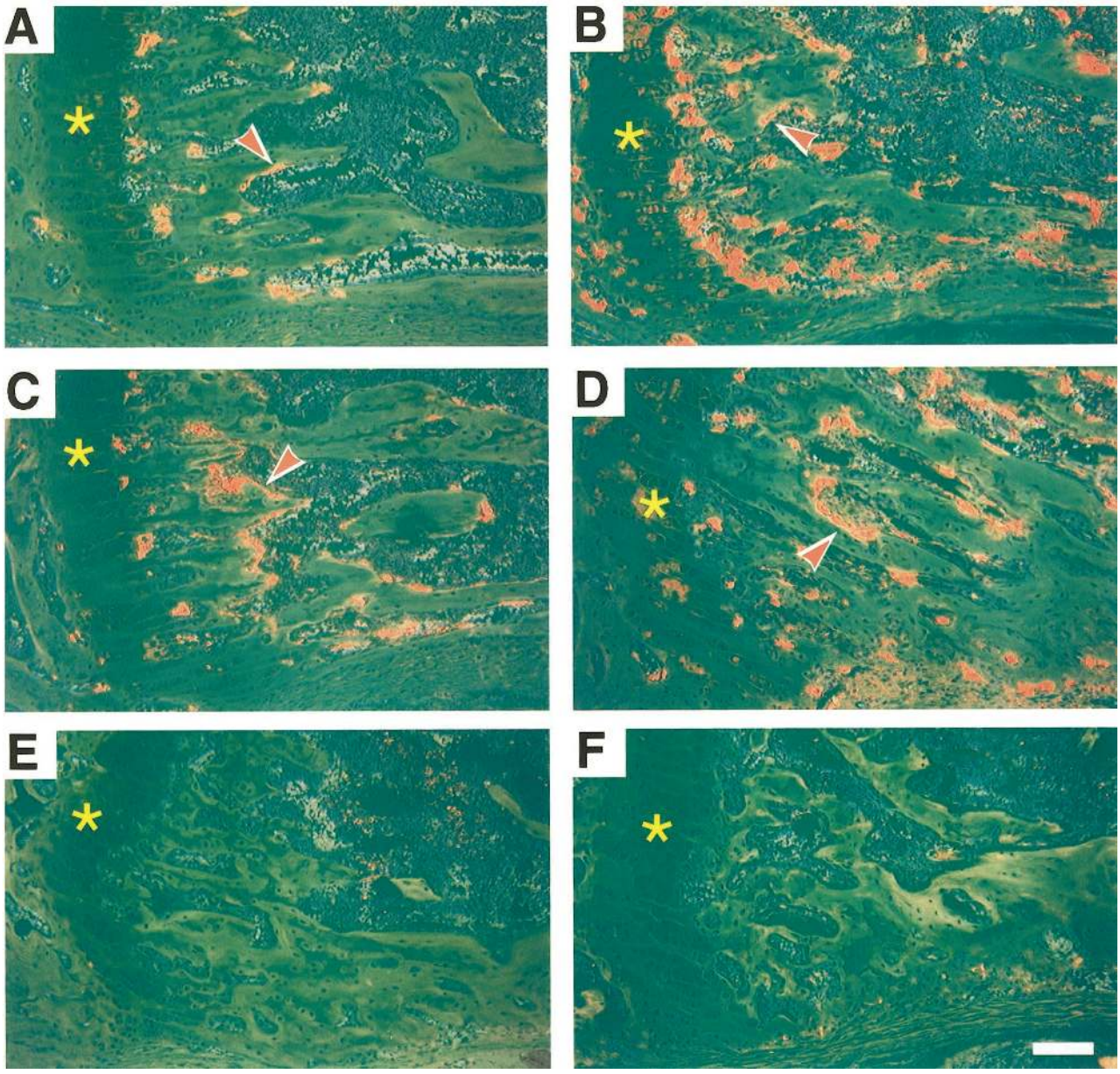


Figure 6. Histology of osteoclasts and apoptotic debris in mice treated with OPGL and OPG. Mice were treated with OPGL and OPG as described in Figure 5. Sections of proximal tibiae were prepared and stained by cathepsin K immunohistochemistry. The immunosections were developed using an alkaline phosphatase substrate (Biotek red), the product of which fluoresces on ultraviolet light exposure. **A–F:** Cathepsin K-immunostained sections of bone from mice treated as follows are shown: saline (7 days) (**A**); OPGL (1 mg/kg, 7 days) (**B**); saline (7 days) → saline (single intravenous dose) → 48 hours (**C**); OPGL (7 days) → saline (single intravenous dose) → 48 hours (**D**); saline (7 days) → OPG (10 mg/kg, single intravenous dose) → 48 hours (**E**); and OPGL (7 days) → OPG (10 mg/kg, single intravenous dose) → 48 hours (**F**). In all cases (**A–F**), the photomicrographs show the junction of the hypertrophic region of the growth plate (denoted by *) with the primary spongiosa. The fluorescent large orange/red, cathepsin K-positive cells (**arrowheads**) are osteoclasts. The scale bar in **F** (applies to **A–F**) represents 100 μm.

OPGL-treated animals that had received saline instead of OPG, osteoclast numbers remained elevated with only a trend toward reduction at the 8-day time point.

Anatomically, the osteoclasts that had regenerated in the OPG-treated animals were located along the metaphyseal side of the growth plate and the periosteal surface of the metaphysis (Figure 8C). These are the same areas that exhibit osteoclast activity in normal animals (Figure 8B) and additionally, exhibit the most evident OPGL mRNA expression by *in situ* hybridization.[3]

To exclude the possibility that OPG exposure *in vivo* had deleterious effects on osteoclast precursor cells, bone marrow collected from animals 48 hours after OPG treatment (10 mg/kg, i.v.) was used to generate osteoclasts *in vitro*. As shown in Figure 8D, the osteoclast-forming potential of bone marrow cells was not impacted by OPG exposure *in vivo*. We also examined bone marrow cells for OPGL-FITC binding 24 hours after exposure to CSF-1 (30 ng/ml) and OPGL (100 ng/ml) and found that the percentage of OPGL-FITC+ cells were virtually iden-

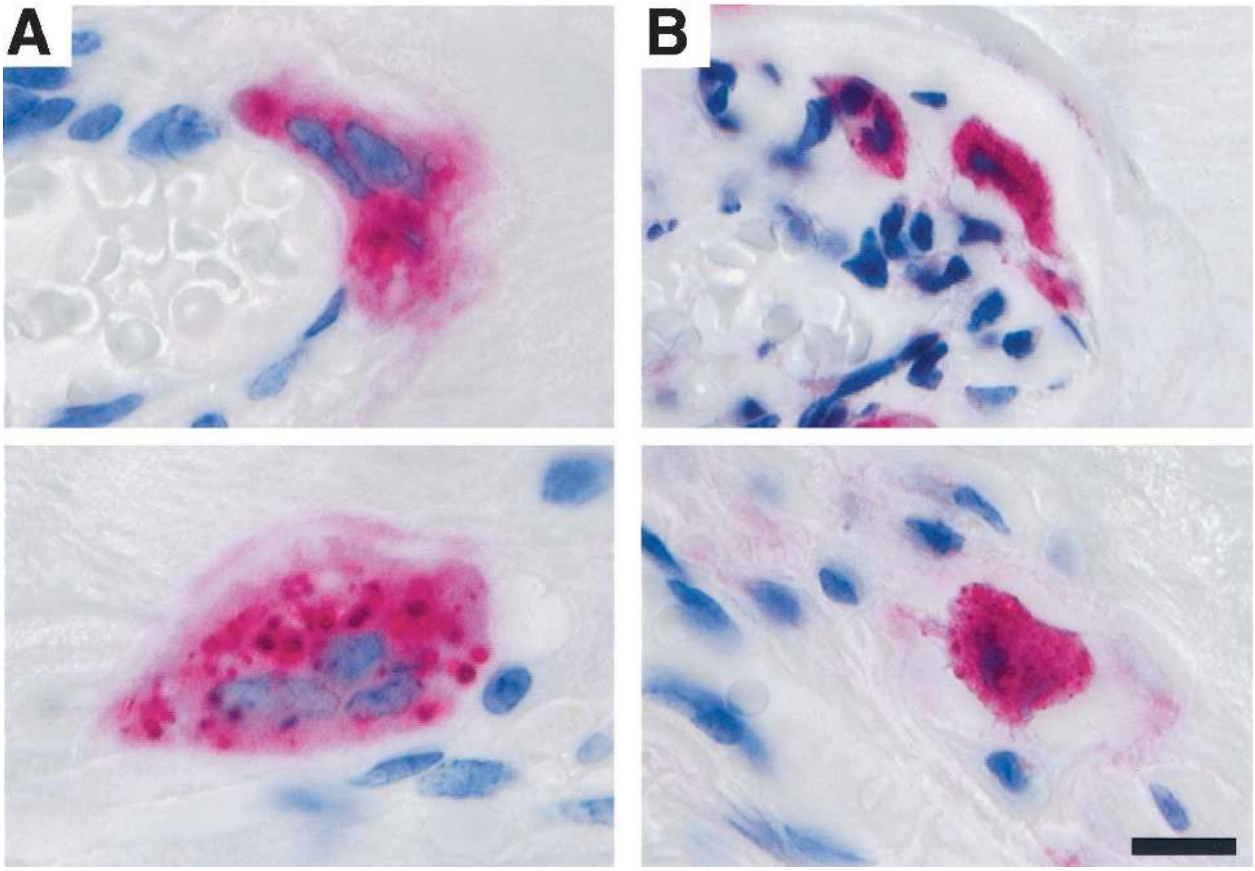


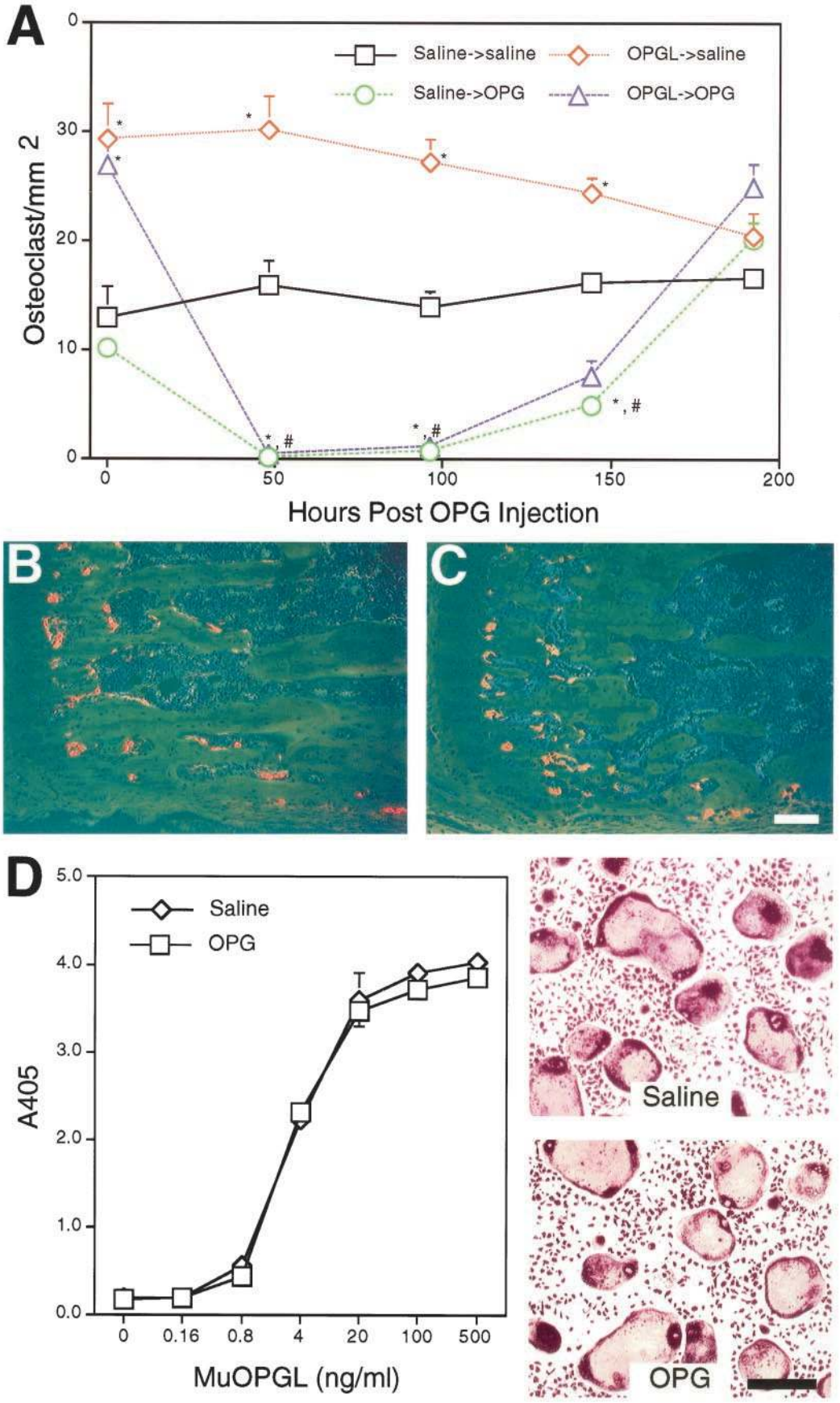
Figure 7. OPG causes osteoclast apoptosis *in vivo*. Mice were treated with OPGL (1 mg/kg, 7 days) as described in Figure 5 and then injected intravenously with a single OPG dose (10 mg/kg) or saline as a control. Tibiae were collected 12 hours after injection and decalcified sections were processed for cathepsin K immunohistochemistry. **A:** Two examples of osteoclasts from bones of saline-treated OPGL-exposed mice are shown. Note the nuclear and cytoplasmic appearance of intact osteoclasts that are closely opposed to bone matrix. **B:** Two examples of osteoclasts from bones of OPG-treated OPGL-exposed mice are shown. Note that the cells have retracted from the bone surface, are shrunken, and exhibit nuclear changes consistent with apoptosis. The scale bar in **B** represents 10 μm.

tical between the saline- (5.43%) and OPG (5.04%)-treated groups. Together these findings indicate that mature osteoclasts are more sensitive to loss of OPGL than their precursor cells.

Discussion

In this study, we have investigated the impact of OPGL withdrawal on osteoclast survival *in vitro* and *in vivo*. In both circumstances, the loss of OPGL stimulation rapidly leads to the disappearance of osteoclasts. Interestingly, the kinetics of both the *in vivo* and *in vitro* loss are similar with changes seen as early as 6 hours and marked changes seen by 24 hours. Additionally, the presence of CSF-1 alone did blunt the rate of osteoclast disappearance *in vitro* consistent with previous reports showing that CSF-1 promotes osteoclast survival,[22,23] but CSF-1 alone was not nearly as effective as the combination of CSF-1 + OPGL. The *in vitro* condition where CSF-1 alone was readded would be somewhat representative of the *in vivo* experiment where OPG was used to treat normal mice. Despite the likely presence of endogenous CSF-1 *in vivo* in these mice, the removal of OPGL activity through intravenously administered OPG led to the obliteration of the osteoclast population *in vivo* within 48 hours of exposure. One must conclude from this observation that endogenous CSF-1 levels in normal mice are insufficient for osteoclast survival in the absence of OPGL. Fortunately, the osteoclast population recovered, but the time to complete recovery was prolonged with return to near normal osteoclast numbers requiring ≥6 days after OPG injection to occur. This delay in osteoclast recovery likely reflects the pharmacokinetics of this form of OPG in mice (mean residence time ~21 hours; S. Martin, unpublished data) as well as the necessity for osteoclasts to regenerate *de novo* from precursor cells. Lastly, this *in vivo* OPG effect likely does not reflect blockade of endogenous TRAIL, another TNF family member to which OPG appears to bind, because TRAIL does not seem to have direct effects on osteoclast formation or osteoclast activity *in vitro* or *in vivo*.[24,25]

Our data differ from Fuller et al[14] and Jimi et al[15] who demonstrate that *in vitro* either OPGL/TRANCE/ODF *or* CSF-1 (M-CSF) alone are sufficient to ensure osteoclast survival. Additionally, we have found that osteoclasts derived from neonatal rat bones cultured on bone slices can survive 24 hours in the absence of OPGL.[13] Methodological differences leading to the presence of contaminating


A
Saline->saline
OPGL->saline
Saline->OPG
OPGL->OPG
Osteoclast/mm 2
Hours Post OPG Injection
B
C
D
Saline
OPG
A405
MuOPGL (ng/ml)
Saline
OPG

cells and the effects of bone matrix constituents on cell survival may in part explain these differing results. The relevance of our observations showing that the combination of CSF-1 + OPGL together determine osteoclast survival are underscored by the *in vivo* experiments that demonstrated that the removal of OPGL through OPG administration led to osteoclast apoptosis. One would predict that administration of sufficient levels of a CSF-1-blocking reagent would also lead to osteoclast apoptosis *in vivo*.

Osteoclast cultures contained mRNA for caspases 1, 2, 3, 6, 7, 8, and 11 based on an RNase protection assay (Figure 3). We have detected caspase 3-like activity in osteoclast cultures and caspase inhibition with either DEVD-FMK or zVAD-FMK had significant anti-apoptotic effects only when combined with OPGL. This suggests that loss of CSF-1 leads to activation of caspase 3, an observation which confirms and extends some of the findings of Okahashi et al.[26] Unlike these investigators, however, we did not find that the use of DEVD-FMK alone improved osteoclast survival in our system. Because these inhibitors did not complement CSF-1 alone, it is probable that a distinct apoptosis pathway may be operative when OPGL is withdrawn from osteoclasts. Whether this pathway involves a caspase (potentially novel) or perhaps a novel apoptosis-inducing, noncaspase protein such as AIF[27] that we have not detected nor searched for in our experiments is unknown.

OPGL signaling in osteoclast precursors and osteoclasts seems to be mediated by RANK and involves TRAFs 2, 5, and 6.[1,17] While not identifying a specific pathway responsible for apoptosis prevention, we have characterized the effects of OPGL and CSF-1 on select signaling pathways in osteoclast cultures. OPGL stimulates both NF-κB and JNK kinase activation just as it seems to do in dendritic cells.[8] In contrast, CSF-1 alone only stimulates NF-κB activity with no effects on JNK kinase. Based on earlier studies, NF-κB activity seems to be essential for osteoclast formation and may play an important role in osteoclast survival.[28] In fact, interleukin 1 has been reported to enhance osteoclast survival *in vitro* through NF-κB activation.[29] While the magnitude of NF-κB activation may be important for osteoclast survival, from our data it is clear that activation of NF-κB alone *per se* is insufficient to promote osteoclast survival.

CSF-1, but not OPGL, enhances the expression of mRNA for both bcl-2 and bcl-X_L, two proteins that are anti-apoptotic.[30] In contrast to its effects on osteoclasts, OPGL/TRANCE does seem to increase bcl-X_L expression in murine dendritic cells.[31] The stimulatory effect of CSF-1 on bcl-2 and bcl-X_L may explain the modest effects that CSF-1 has on osteoclast survival (Figures 1 to 3). On the other hand, OPGL, but not CSF-1, maintained c-src mRNA at elevated levels. Obviously elevated c-src alone was not sufficient to prevent osteoclast apoptosis. From these data one must conclude that the mechanism able to most effectively prevent osteoclast apoptosis relies on the combined signaling pathways of OPGL and CSF-1. Additionally, as some of the genes induced by OPGL on osteoclasts include proteins with signaling pathways of their own (c-src, β3 integrin for instance), it is possible that these signaling pathways may also synergize with those driven by CSF-1. Identification of pivotal intersects in these pathways may yield known or novel molecules that would present logical targets for innovative therapeutic strategies.

In a broader context, these data provide additional insights into how the osteoblast may modulate both the beginning and end of the resorption component of bone remodeling. At resorption initiation, osteoblast presentation of OPGL and CSF-1, perhaps attended by down-regulated OPG production, promotes recruitment, and activation of osteoclasts and their precursors. As the stimulus for resorption abates, osteoblasts reduce the supply of available OPGL through OPGL down-regulation, increased OPG production, or perhaps some combination of the two. The loss of OPGL immediately blocks further osteoclast differentiation from precursor cells and provides a potential death signal for previously formed osteoclasts. These events likely are anatomically quite specific as both OPGL and OPG are local factors because of osteoblast membrane expression and matrix affinity, respectively. Lastly, the capacity for a single, pharmacologically administered dose of OPG to cause rapid, massive osteoclast apoptosis provides both a potent mechanism for some of its *in vivo* activity and a strong rationale for its use as an anti-resorptive therapeutic.

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Figure 8. Effects of OPG treatment on osteoclast recovery *in vivo* and osteoclast precursors *in vitro*. Mice were treated as in Figure 5 except that mice were analyzed at 0, 48, 96, 144, and 192 hours after a single injection of saline or OPG. **A:** Quantitative histomorphometry of cathepsin K-immunostained sections of distal femur were analyzed for osteoclast numbers as described in Figure 5. Different from saline → saline group, *$P < 0.01$; different from OPGL → saline group, **#**, $P < 0.01$. Histological sections of the proximal tibiae stained with cathepsin K antibodies from saline → saline treated or OPGL → OPG treated mice are shown in **B** and **C**, respectively. The scale bar represents 100 μm. Note that osteoclast numbers recover between 144 and 192 hours after OPG exposure and that the osteoclasts re-appear in the same anatomical locations in the OPG-treated animals as they are observed in the saline-treated animals. **D:** For osteoclast precursor analysis, bone marrow from mice treated with either saline or OPG were used in the *in vitro* osteoclast forming assay. The **left panel** shows the result of the TRAP solution assay (mean ± SD of A405 values from triplicate cultures) and the **right panels** show the cytomorphology of TRAP-stained cultures that were differentiated with CSF-1 (30 ng/ml) and OPGL (100 ng/ml). The labels "saline" and "OPG" reflect the *in vivo* treatments of the mice before bone marrow isolation and the scale bar represents 200 μm. Note that *in vivo* OPG exposure has no inhibitory effects on the ability of bone marrow osteoclast precursors to differentiate into osteoclasts *in vitro*.

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